

**Potential immunomodulatory effects of SCAP on Treg conversion in tissue regeneration
for regenerative endodontic treatment**

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Abstract

Aim To evaluate the expression of Foxp3-positive lymphocytes around newly formed tissue after regenerative endodontic treatment (RET) *in vivo* and investigate the effects of stem cells from apical papilla (SCAP) on the conversion of CD4⁺CD25⁻T cells to CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs) *in vitro*.

Methodology Three 6-month-old beagles with nine doubled root premolars of each dog were randomly assigned to the RET group and the control group. RET was performed after apical periodontitis had been induced in the experimental immature teeth. Three months later, the expression of Foxp3 was detected in the histological sections by immunofluorescent staining. Human SCAP and CD4⁺CD25⁻T cells from mice spleens (1:1 and 1:5) were co-cultured in cell-cell contact or in Transwells respectively for 24 and 72 h *in vitro*. The percentage of Tregs was evaluated by flow cytometry. The results were analysed using Fisher's exact test and analysis of variance. $P < 0.05$ was regarded as statistically significant.

Results Inflammatory cells were present with tissue regeneration in the RET group, and Foxp3-positive T cells were enriched around the newly formed tissues. SCAP promoted Treg conversion after 72 h *in vitro*. Cell-cell contact played an important role after the 24 h co-culture, while soluble factors were

also involved after 72 h ($P < 0.05$).

Conclusions SCAP promoted the conversion of pro-inflammatory T cells to Tregs *in vitro*. Tregs were enriched around the regenerating tissues in the root canals after RET, which may create a suitable immune microenvironment for the differentiation of SCAP. This study provides an underlying mechanism for tissue regeneration during RET.

Introduction

Regenerative endodontic treatment (RET) is a new option for immature permanent teeth with pulp necrosis that eliminates clinical symptoms and furthers root development (Kim *et al.* 2018). RET is a biological method based on the concept of the triad of tissue engineering (Nakashima & Akamine 2005). It is widely accepted that mesenchymal stem cells (MSCs) are recruited into the root canal after evoked-bleeding during RET, and supported by the blood clot as a scaffold, with the help of growth factors released by blood platelets and dentin to proliferate and differentiate into new tissues (Hargreaves *et al.* 2013). Although RET has been performed in many clinical cases, the biological mechanisms of RET remain unclear.

In a previous animal study, lengthening of the root, thickening of the root canal wall and closure of the apex were achieved after RET. The histological findings showed that various tissues regenerated in the root canal, such as free cellular cementum-like tissues and pulp-like tissues. Interestingly, lymphocytes were present in some root canals around the newly formed tissues (Zhang *et al.* 2014). However, there were no clinical symptoms and no radiolucency after RET. Thus, an explanation of why there were lymphocytes around the regenerated tissues after RET and what their possible roles were in tissue regeneration is required.

The local microenvironment in the apical area, particularly the immune components plays an important role in tissue regeneration (Leprince *et al.* 2012). T cells are a vital part of the local microenvironment. T cells traditionally include CD4⁺ helper T cells and CD8⁺ cytotoxic T cells. CD4⁺ T cells are divided into two categories of CD4⁺CD25⁻effector T cells and CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs). Effector T cells respond to a vast array of pathogens; however, excessive inflammatory cytokines secreted by these effector T cells cause pathological damage or affect tissue regeneration (Hall 2015). Tregs are a specialized subpopulation of T cells. Foxp3 is an indispensable transcription factor important for Tregs development and function, and it has been used as a Tregs biomarker (Hori *et al.* 2003). Tregs are produced in the thymus or converted from naive T cells in the peripheral environment, which is called Treg conversion (Chen *et al.* 2003). Tregs are regarded as anti-inflammatory cells that play a vital role in maintaining immune homeostasis (Lee & Lee 2018), and can facilitate regeneration of various tissues by creating a beneficial immune microenvironment *via* immune cells modulation or by directly activating progenitor cells (Li *et al.* 2018). Recently, several studies have shown that MSCs upregulated Tregs, which served for regulating the local or systemic inflammation response to promote tissue regeneration (Wu *et al.* 2014, Liu *et al.* 2015). The increased ratio of Tregs and enhanced immune suppression function helps to create an immune microenvironment

contributing to tissue regeneration (Lei *et al.* 2015). Therefore, whether the lymphocytes that appeared during RET were Tregs and from where did they originate needs to be clarified.

Stem cells from the apical papilla (SCAP) are one of the most important cell sources for tissue regeneration during RET (Sonoyama *et al.* 2006, 2008). SCAP may survive and maintain their stemness in a case of advanced pulpal necrosis and apical periodontitis (Chrepa *et al.* 2017). Moreover, SCAP have low immunogenicity and possess immunomodulatory properties. SCAP can effectively regulate the biological function of T cells after co-culture with autologous or allogeneic peripheral blood monocytes. It has been reported that SCAP failed to stimulate allogeneic T-cell proliferation and suppressed T cell proliferation in an apoptosis-independent manner *in vitro* (Ding, *et al.* 2010, Ding, *et al.* 2010). However, the capacity of SCAP on Treg conversion has not yet been reported.

In the animal study in which RET was performed in beagles, regeneration of various tissues in the root canal occurred. Lymphocytes had infiltrated around the newly formed tissues in some root canals. The aim of this study is to evaluate further the expression of Foxp3 to reveal whether Tregs were involved after RET in beagles. In addition, SCAP and CD4⁺CD25⁻ T cells were co-cultured to investigate whether SCAP played an immunomodulatory role to promote Treg conversion *in vitro* to gain a further understanding of the underlying mechanisms of RET.

Materials and Methods:

Animals

Six-month-old beagles were purchased from Shenyang Kangping Institute of Laboratory Animals (Shenyang, China). C57BL/6J mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All animal experiments were approved by the Ethics Commission of China Medical University, Shenyang, China (15008M).

Three six-month-old beagles with nine incompletely developed premolars, two roots each, were used in this experiment. Nine premolars of each dog were randomly divided into 2 groups, 6 teeth in the experimental group and 3 teeth in the control group. Therefore, there were 36 roots in the experimental group ($n = 36$) and 18 roots in the control group ($n = 18$). Healthy immature permanent teeth were used as control to compare the continued root development after RET with the physiological root maturation. All surgical procedures were performed under general anaesthesia with an intramuscular injection of 0.5 mg/kg pentobarbital sodium (Merck, Darmstadt, Germany) supplemented with local anaesthesia. The animals received 2 mg/kg ibuprofen postoperatively. The apical periodontitis animal model was established, the root canal was disinfected and RET was performed as described previously (Zhang *et al.* 2014). In brief, the dental pulp was mechanically exposed and

disrupted. A cotton pellet soaked with supragingival plaque was placed in the pulp chamber and the access cavity was restored. The apical periodontitis animal model was established with apparent apical radiolucencies after about 3 weeks. The canals were disinfected by irrigation and medicated with a triple antibiotic paste. Last, the periapical tissue was stimulated to bleed to form a blood clot and the access cavity was sealed.

Histological analysis

The experimental animals were euthanized 3 months later by perfusion with 10% neutral buffered formalin. The jaws were dissected and fixed in 10% formalin and decalcified in 10% EDTA (Decal Chemical Corp., Congers, NY, USA). The specimens were embedded in paraffin and sectioned every 5 μm . The sections were deparaffinized and stained with haematoxylin and eosin (HE) and observed under a light microscope. The sections were covered with the anti-Foxp3 antibody (1:50, Abcam, Cambridge, MA, USA) in a humid chamber and incubated overnight at 4°C for immunofluorescence staining. FITC-conjugated rabbit anti-goat secondary antibody (1:200, Zsbio Commerce Store, Beijing, China) was added to each tissue section and incubated at 37°C for 30 min. Nuclei were stained with DAPI. Slides were observed under a fluorescence microscope.

Primary isolation and culture of SCAP

Volunteers were recruited from the outpatient operating room at the Affiliated Stomatological Hospital of China Medical University. Informed consent was obtained from patients or parents (patients < 18 years old). This experiment was approved by the Ethics Committee of the School of Stomatology, China Medical University, Shenyang, China (201315). SCAP were primary cultured and characterized as described previously (Bi *et al.* 2018). The apical papilla was digested with 3 mg/mL Collagenase I (Worthington Biochemical Co., Lakewood, CO, USA) and 4 mg/mL Dispase II (Boehringer Ingelheim, Mannheim, Germany). Cells were seeded with growth medium containing the alpha modification of Eagle's medium (HyClone, Logan, UT, USA) supplemented with 15% foetal bovine serum (ExCell Bio, Shanghai, China), 100 mM L-2 ascorbic acid phosphate (Sigma-Aldrich, St. Louis, MO, USA) and 100 U/mL penicillin/streptomycin (HyClone). Cells at passages 3–5 were used for subsequent experiments.

Surface markers expressed on SCAP by flow cytometry

SCAP at the third passage were adjusted to $1 \times 10^6/\text{mL}$. Anti-human CD73 antibody labelled with PE and anti-human CD31, CD34, CD90, CD105 and CD146 (1:100, Abcam) antibody labelled with FITC were used to detect the surface marker expression on SCAP. SCAP were incubated with CD31, CD34, CD73, CD90, CD105 and CD146 antibodies respectively on ice for 30 min. The expression of each

surface marker on SCAP was tested by flow cytometry (Becton-Dickinson, Islandia, NY, USA).

Multilineage differentiation assay

SCAP at the third passage were seeded into six-well plates and cultured to 80% confluence.

Osteogenesis culture medium supplemented with 1.8 mM monopotassium phosphate (Sigma-Aldrich)

and 10 nM dexamethasone (Sigma-Aldrich) was exchanged for 6 weeks. The specimens were stained

with 1% Alizarin red S. The adipogenic differentiation assay included adipogenesis culture medium

with 500 μ M isobutyl-methylxanthine (Sigma-Aldrich), 0.5 μ M hydrocortisone (Sigma-Aldrich), 60

μ M indomethacin (Sigma-Aldrich) and 10 μ M insulin (Sigma-Aldrich) replaced two or three times per

week for 3 weeks. Oil red O was used to stain the oil droplets.

Immunomagnetic isolation of CD4⁺CD25⁻ T cells from mice spleen

All 8–12 weeks old C57BL/6 female mice were sacrificed under anaesthesia. The spleen was minced

to obtain a single cell suspension. CD4⁺CD25⁻ T cells were negatively immunomagnetically

isolated twice using a CD4⁺CD25⁺ regulatory T cell isolation kit (Miltenyi Biotec GmbH, Bergisch

Gladbach, Germany) according to the manufacturer's instructions. First, the non-CD4⁺ T cells were

magnetically labeled. Then the labeled non-CD4⁺ T cells and unlabeled CD4⁺ T cells were separated

with a MACS[®] column (Miltenyi Biotec GmbH). In the second step, the CD25⁺ PE-labeled cells were

magnetically labeled. The labeled CD4⁺CD25⁺ T cells and unlabeled CD4⁺CD25⁻ T cells were separated, and then CD4⁺CD25⁻ T cells were collected for further assays. After isolation, T cells were incubated with PerCP-conjugated anti-CD4 and allophycocyanin-conjugated anti-CD25 antibodies (eBioscience, San Diego, CA, USA) (2 µg/mL each). Cells characterized as CD4 positive and CD25 negative were evaluated by flow cytometry. The percentage of CD4⁺CD25⁻ T cells represented the purity of CD4⁺CD25⁻ T cells after magnetic isolation. Cells with purity > 90% were used in the following experiment. CD4⁺CD25⁻ T cells were cultured for 48 h in complete Dulbecco's modified Eagle's medium (HyClone) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 55 mM 2-mercaptoethanol (Solarbio, Beijing, China), 100 U/mL penicillin/streptomycin (HyClone).

Co-culture of SCAP and CD4⁺CD25⁻ T cells

T cell activation is a key regulatory point for adaptive immune responses that requires an initial activation signal provided by CD3 and a costimulatory signal provided by CD28 (Frauwirth & Thompson 2002). Therefore, CD4⁺CD25⁻ T cells were pre-cultured in T cell culture medium in the presence of plate bound anti-CD3ε antibody (BioLegend, San Diego, CA, USA) (2 µg/mL) and soluble anti-CD28 antibody (BioLegend) (2 µg/mL) for 48 h according to Chen *et al.* (2013). SCAP (2 × 10⁵ per well) were seeded in a 12-well plate and cultured for 24 h. Both cell-cell contact and Transwell

manner were used to test the effect of SCAP on the Treg conversion. Activated CD4⁺CD25⁻ T cells ($2 \times 10^5/1 \times 10^6$) were co-cultured directly in the cell-cell contact group, in which SCAP directly affected the T cells. A polyethylene terephthalate hanging cell culture insert with a pore size of 0.4 μm (Merck KGaA, Darmstadt, Germany) was used to separate the SCAP and T cells in the Transwell group. Two different ratios of SCAP: CD4⁺CD25⁻ T cells (1:1 and 1:5) and two time points (24 and 72 h) were evaluated in this study. The T cells were gently collected, and the percentages of CD4⁺CD25⁺ T cells and CD4⁺CD25⁺Foxp3⁺ T cells were measured by flow cytometry.

Staining of Tregs by flow cytometry

Cells (1×10^6) were treated with PerCP-conjugated anti-CD4, allophycocyanin-conjugated anti-CD25 antibodies (eBioscience) (2 $\mu\text{g/mL}$ each). After cell fixation and permeabilization (eBioscience), the cells were stained with R-phycoerythrin-conjugated anti-Foxp3 antibody (eBioscience) (3 $\mu\text{g/mL}$). The percentage of Tregs was evaluated by flow cytometry. CD4⁺ T cells were gated firstly, and then T cells labeled with CD25 positive, Foxp3 positive and CD25 Foxp3 double positive were sorted. Thus, the percentages of CD4⁺CD25⁺ T cells and CD4⁺CD25⁺Foxp3⁺ T cells (Tregs) were calculated and evaluated.

Statistical analysis

The statistical analysis was performed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). All roots were observed independently in the animal experiment. The lymphocyte infiltration data were analysed statistically with Fisher's exact test. The *in vitro* data were reported as mean \pm SD. The quantitative data were tested to show normal distribution. Analysis of variance was used to analyse the differences among the cell-cell contact group, the Transwell group, and the control group. A post-hoc multiple comparison test was conducted to compare the differences between each two groups. A *P*-value < 0.05 was considered significant.

Results

Foxp3-positive T cells are enriched around the newly formed tissue after RET

After the treatment, root lengthening, root canal wall thickening, and apical apex closure were observed on radiographs (Fig. 1a, b). Some specimens were damaged during the sectioning process. There were 29 roots in the RET group available for histological observation. Pulp-like tissues and free cellular cementum-like tissues in the root canal and cellular cementum-like tissues on the root wall were observed. Inflammatory cells were present in the periapical area and root canal together with newly formed tissue, and most were lymphocytes (Fig. 2a, b). Fisher's exact test was used to compare the difference between the ratio of lymphocytes infiltration in the RET and the control group. The ratio of

lymphocyte positive specimens was 89% (26 of 29 specimens in the RET group had inflammatory cells).

No inflammatory cells were observed in the control group (Fig. 2c, d). The difference is significant ($P < 0.001$). This result showed that the immune cells and/or the local immune microenvironment might participate in the tissue regeneration after RET.

To identify whether Tregs were involved in the local immune microenvironment, the immunofluorescence staining was used to detect the expression of mature Treg marker Foxp3. Foxp3-positive cells were enriched around the newly formed tissue in the RET group (Fig. 3a); while no Foxp3-positive cells were detected in the root canals of the control group (Fig. 3b). These data indicated that Tregs were involved in RET, and might have promotion effects on the tissue regeneration.

SCAP promoted CD4⁺ T cell conversion to CD4⁺CD25⁺Foxp3⁺ T cells

To further explore the detailed mechanisms of enriched Tregs around the newly formed tissue after RET, a SCAP/CD4⁺CD25⁻ T co-culture system was used to explore whether SCAP have immunomodulatory effects on the Treg conversion *in vitro*. SCAP had spindle-shaped (Supplementary Fig. 1a) and possessed osteo/adipogenic differentiation potential (Supplementary Fig. 1b, c). Flow cytometry revealed that SCAP expressed MSC markers (CD73, CD90, CD105, and CD146), but failed to express hematopoietic stem cell markers (CD31 and CD34) (Supplementary Fig. 1d). CD4⁺CD25⁻ T

cells were magnetically isolated with the purity > 90%. After a 24 h co-culture, the percentage of only CD4⁺CD25⁺ T cells was upregulated in the cell-cell contact group when compared with the control and Transwell groups (Fig. 4a, b). However, there was no difference in the percentage of CD4⁺CD25⁺Foxp3⁺ T cells between the SCAP treated group and control groups, no matter the low cell ratio (1:1) or the high cell ratio (1:5). After a 72 h co-culture, the percentage of CD4⁺CD25⁺Foxp3⁺ T cells was upregulated in the cell-cell contact and the Transwell groups compared to the control group (Fig. 4c, d). Furthermore, the percentage of CD4⁺CD25⁺Foxp3⁺ T cells in the cell-cell contact group was higher than the Transwell group. The same tendency was observed for the low cell ratio and the high cell ratio groups. Collectively, these data showed that cell-cell contact played an important role in the early stage (24 h) of the promotion of SCAP on Treg conversion, and paracrine effects were also involved in the late stage (72 h). SCAP promoted CD4⁺ T cell conversion to CD4⁺CD25⁺Foxp3⁺ T cells *in vitro*.

Discussion

The primary goal of RET is elimination of the clinical symptoms and periapical inflammation. The secondary goal is further root maturation (Kim *et al.* 2018). The preliminary aim of this animal study was to compare continued root development after RET with the physiological root maturation.

Therefore, the limited number of samples was only divided into two groups, the experimental RET group and the healthy control group. The infiltration of T lymphocytes was found mainly Foxp3 positive Tregs in RET group based on histological observations. Some studies have reported that continued root maturation and infiltration of lymphocytes in the root canal appeared simultaneously (Wang *et al.* 2010, Londero *et al.* 2015). The immune system or immune microenvironment including lymphocytes has a close association with initiation, progress and termination processes of tissue regeneration (Liu *et al.* 2011, Leprince *et al.* 2012, Godwin *et al.* 2013, Ayyaz *et al.* 2015). There is a dynamic balance between T lymphocyte subpopulation in the development of apical periodontitis. Effector T cells predominated during the active phase of lesion development, whereas suppressor T cells were outnumbered effector T cells in the chronic phase (Stashenko & Yu 1989). It is speculated that an unfavourable situation persists before the RET treatment, while after treatment, the upregulated Tregs release anti-inflammatory cytokines and pro-healing growth factors contributing to tissue repair. Therefore, the lymphocytes that appeared during RET may serve as a part of suitable microenvironment for new tissue formation (Wang *et al.* 2010). However, there is a limitation of the *in vivo* study due to the lack of a positive control group (apical periodontitis without treatment). We can't tell clearly whether the Tregs are present due to inflammation and/or due to regeneration process from this animal study. Further study

on the expression of Tregs at different time points before and after RET would provide a deeper understanding of the immunologic mechanism of regenerative endodontics.

Accordingly, stem cells in a favourable microenvironment tend to migrate, proliferate and differentiate in the periapical area of immature permanent teeth. This microenvironment is composed of growth factors, anti-inflammatory immune cells and cytokines. Tregs are a vital component of this environment (Leprince *et al.* 2012). The anti-inflammatory and anti-apoptotic capacities of Tregs promote successful tissue repair and regeneration, which is associated indirectly with the effector immune response and directly with tissue cell functions (Lei *et al.* 2015). Systemic infusion of Tregs markedly reduces IFN- γ and tumour necrosis factor- α levels and improves bone marrow mesenchymal stem cell (BMMSC)-based bone regeneration in calvarial bone defect of C57BL6 mice (Liu *et al.* 2011). Lei *et al.* (2015) reported that Tregs act directly on osteoblasts or other MSCs through coordination of the CD39-CD73-(adenosine)-ADOR pathway to promote differentiation of MSCs and facilitate tissue regeneration. Immunofluorescent staining was used in the present study and Foxp3-positive cells around the newly formed tissue were detected. Therefore, Tregs could directly or indirectly participate in the tissue regeneration of RET. However, the origin of Tregs in RET remains unknown. Thus, *in vitro* experiments were carried out to gain further understanding.

Tregs are mainly divided into two categories based on developmental origin. One is called tTreg or nTreg, which develop and mature in the thymus. The other is pTreg or iTreg, which develop from naive CD4⁺ T cells in the periphery outside of the thymus (Lee & Lee 2018). Many studies have shown that MSCs can either increase the number and function of Tregs or promote the conversion of naive CD4⁺ T cells into Tregs (Takahashi *et al.* 2014, Wang *et al.* 2015). Liu *et al.* (2015) reported that systemic infusion of BM-MSCs markedly enhances bone regeneration by upregulating Tregs. The anti-inflammatory and immunosuppressed properties of MSCs in the periapical area may also contribute to tissue regeneration and wound healing (Zhang *et al.* 2013, Araujo-Pires *et al.* 2014). MSCs possess low immunogenicity (Zhang *et al.* 2015). Animal studies have been conducted on human dental stem cells to treat autoimmune disorders of mice. In addition, studies on the immunoregulatory properties of dental stem cells have been conducted with human dental stem cells and mice spleen immune cells co-culture system *in vitro* (Zhao *et al.* 2012, Liu *et al.* 2014). Therefore, SCAP, the most important stem cell during RET, were used to co-culture with mice CD4⁺CD25⁻ T cells *in vitro* to explore whether SCAP promoted Treg conversion.

No difference in the percentage of Tregs was detected after the 24 h co-culture. However, the expression of CD25 increased significantly in the cell-cell contact group. The expression of Foxp3 and

CD25 was up-regulated after the 72 h co-culture both in the cell-cell contact group and the Transwell group. CD25 is one of the three different components of the IL-2 receptor, which combines with the other two chains to form a complex with high affinity to regulatory T cells (Minami *et al.* 1993, Hémar *et al.* 1995). It is a potentially putative permissive factor for Foxp3 expression (Lio & Hsieh 2008, Guo *et al.* 2013). In the peripheral environment, the first step leads to the generation of CD4⁺CD25⁺Foxp3⁻ cells containing iTreg cell precursors. Foxp3 was induced in the next step to obtain iTregs. These iTregs with relatively stable expression of Foxp3 exert their immunosuppressive properties to further maintain immunotolerance (Guo *et al.* 2013). Taken together, these results suggest that SCAP promote the Treg conversion *in vitro*. Cell-cell contact played an important role during the early stage (24 h) and paracrine effects were involved in Treg conversion by SCAP at the later stage (72 h). However, the detailed mechanisms of the Treg conversion by SCAP and how these two cell types work *in vivo* are topics of further investigation.

In the animal study, the correlation between the expression of Tregs and the amount of tissue regeneration was difficult to evaluate quantitatively from the histological sections because of the complexity of newly formed tissues in the root canal. And the exact role of Tregs in the regeneration process is still unknown. Further experiments are needed to demonstrate whether and how Tregs

contribute to tissue regeneration *in vivo*.

Conclusion

SCAP promoted T cell conversion to Tregs. Tregs were enriched around regenerating tissues in the root canals after RET, which may create a suitable immune microenvironment for tissue regeneration. The study discloses the immunomodulatory properties of SCAP, which play an important role in clinical RET. The exact role of Tregs in the regeneration process and the possible interaction between SCAP and Tregs in RET need further investigation.

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Conflict of interest

The authors declare no potential conflicts of interest in this article.

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Figure legends

Figure 1 Radiographic manifestations of immature permanent teeth affected with apical periodontitis before and after regenerative endodontic treatment (RET)

(a) Radiographic manifestations of immature permanent teeth after apical periodontitis; incomplete root formation and apparent periapical radiolucency. (b) Radiographic manifestations of immature permanent teeth after RET; healing of periapical radiolucencies, apical closure and root wall thickening. Yellow arrowheads indicated the apical areas of teeth before RET. Blue arrowheads indicated the apical areas of teeth after RET.

Figure 2 Postoperative inflammatory cell infiltration in the root canal after regenerative endodontic treatment (RET)

(a) Histological observation showed inflammatory cells in the root canal of the RET group; most were lymphocytes. (b) Magnified view of the boxed region in (a). (c) Histological observation showed no inflammatory cells in the pulp of the control group. (d) Magnified view of the boxed region in (c). Blue arrowheads indicated lymphocytes. D, dentine. P, pulp. PDL, periodontal ligament. Scale bars = 200 μm (a, c); and 100 μm (b, d).

Figure 3 Foxp3-positive cells are present around newly formed tissue

(a) Immunocytofluorescent staining showed a large number of Foxp3-positive cells in the root canal of the regenerative endodontic treatment (RET) group; white arrowheads indicated a Foxp3-positive cells.

(b) Immunocytofluorescent staining showed no Foxp3- (green) positive cells in the pulp of the control group. Scale bar = 100 μ m.

Figure 4 Stem cells from apical papilla (SCAP) upregulate the expression of CD25 and Foxp3

(a, b) Flow cytometric analysis of SCAP and CD4⁺CD25⁻ T cells (SCAP:CD4⁺CD25⁻ T cells = 1:1 or 1:5) co-cultured for 24 h showed that SCAP promoted CD25 expression in the cell-cell contact group but SCAP had no significant effect on the expression of Foxp3. (c, d) Flow cytometric analysis of SCAP and CD4⁺CD25⁻ T cells (SCAP:CD4⁺CD25⁻ T cells = 1:1 or 1:5) co-cultured for 72 h showed that SCAP promoted CD25 and Foxp3 expression both in the cell-cell contact and the Transwell groups. Foxp3 expression was higher in the cell-cell contact group than in the Transwell group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Error bars: mean \pm SD.

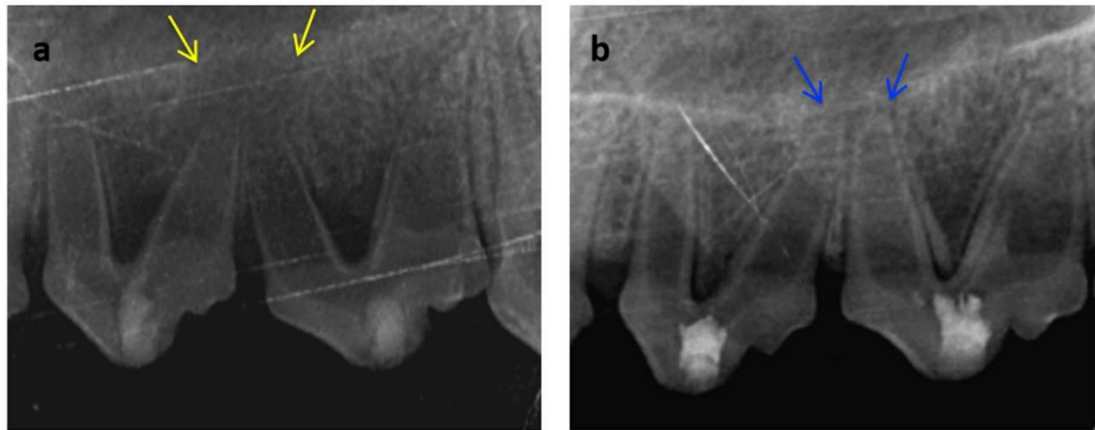


Figure 1: Radiographic manifestations of immature permanent teeth affected with apical periodontitis before and after regenerative endodontic treatment (RET).

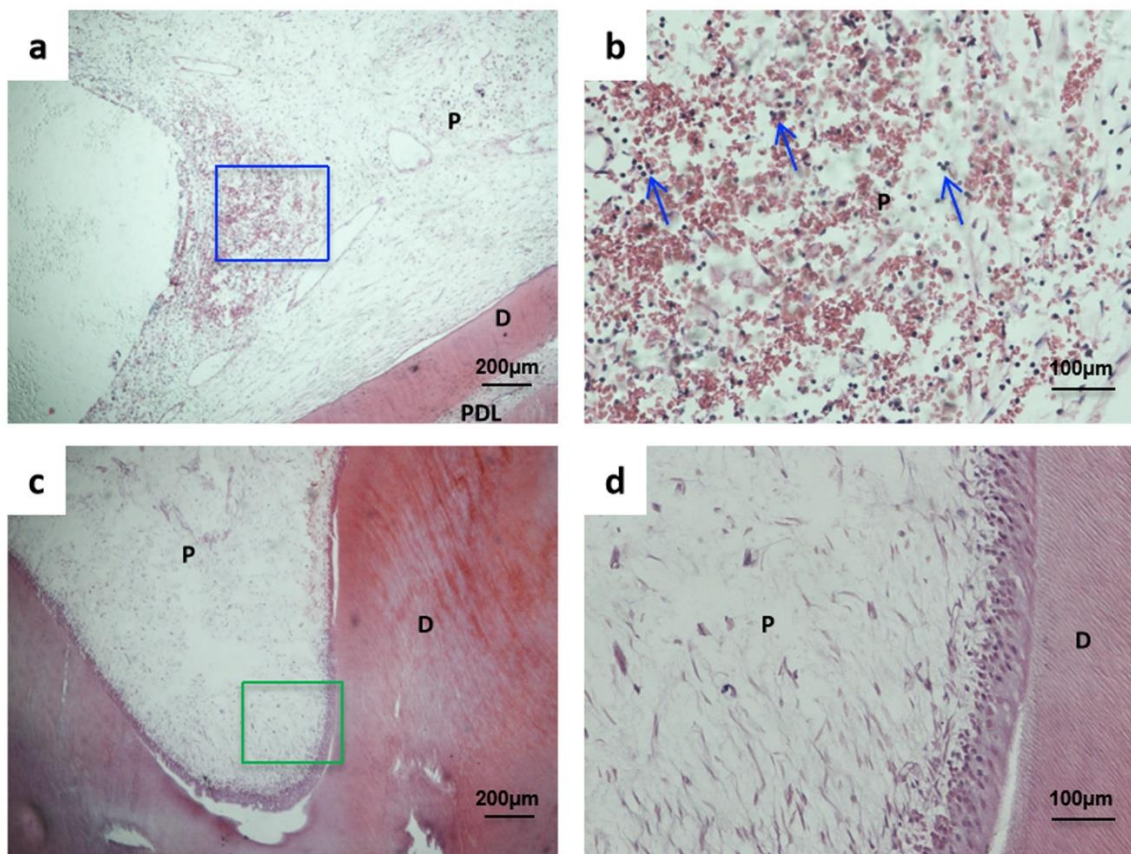


Figure 2: Postoperative inflammatory cell infiltration in the root canal after regenerative endodontic treatment (RET).

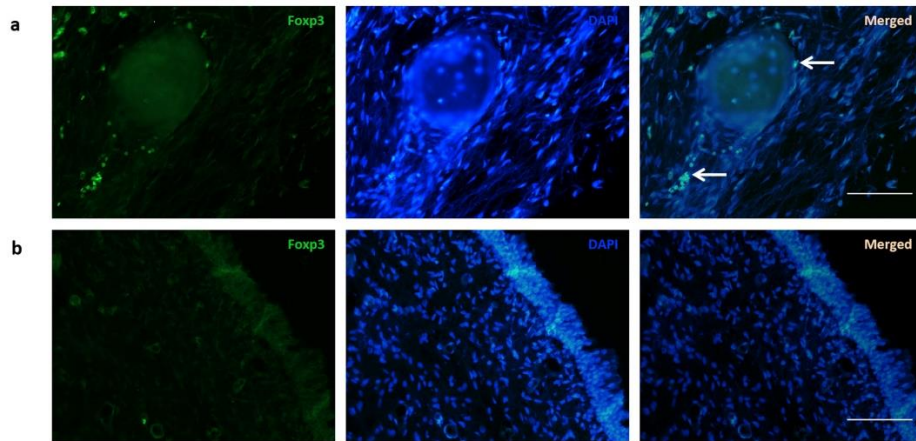


Figure 3: Fxp3-positive cells are present around newly formed tissue.

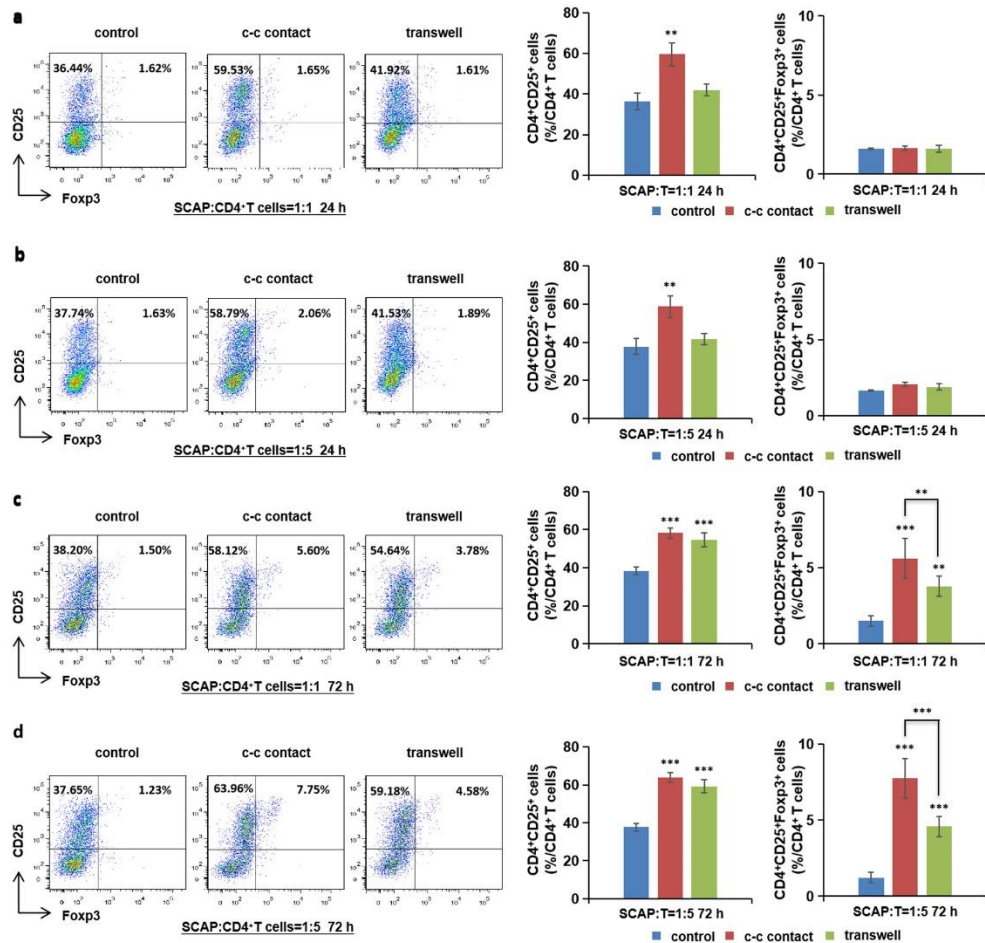
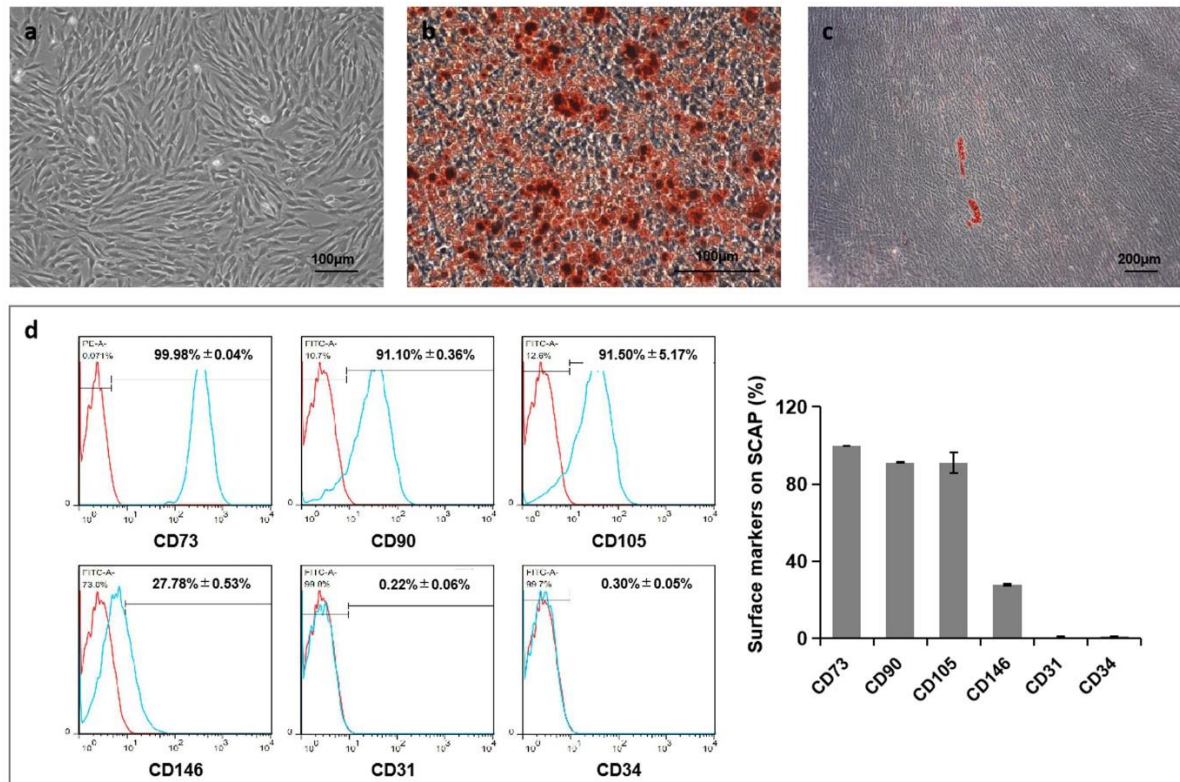


Figure 4: Stem cells from apical papilla (SCAP) upregulate the expression of CD25 and Fxp3.



Supplementary Figure 1. Primary culture and characterization of SCAP.

(a) Morphologically, SCAP was spindle-shaped under the inverted phase contrast microscope. (b) SCAP formed Alizarin Red S positive mineralized nodes after osteogenic induction. (c) SCAP formed Oil Red O positive lipid clusters after adipogenic induction. (d) Flow cytometric analysis showed SCAP expressed mesenchymal stem cell surface markers (CD73, CD90, CD105 and CD146), but failed to express hematopoietic stem cell surface markers (CD31 and CD34). Scale bars = 100 μ m (a, b); and 200 μ m (c).